3836-Pos Board B564
Opening and Closing of the HCN2 Channel Pore is Voltage-Independent
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Recent studies have suggested that the opening and closing of Hyperpolarization-activated Cyclic Nucleotide-gated 'HCN' channels involve a step that is voltage-independent, which depends upon a region that resides within the S4 and S6 transmembrane domains of the channels. In the current study, we use two electrode voltage clamp of the HCN2 channel expressed in *Xenopus* oocytes to show that mutation of a residue site in the S6 segment, in region that likely corresponds to the pore, dramatically slows channel closing, without large effects on the rate of channel opening, and decreases the dependence of channel closing on voltage. A 6-state,but not a 4-state, cyclic allosteric model incorporating voltage-dependent transitions moving the channels between resting and active states and voltage-independent transitions between closed and open states was best able to describe the complex gating behaviour of both the wild type and pore mutant channels in response to changes in voltage. Modification of the voltage-independent closing transition recapitulates both the very slow and voltage-independent closing of mutant channel. In summary, our data supports a cyclic allosteric model of HCN2 channel gating in which the opening and closing of the pore is voltage-independent.

3837-Pos Board B565
Isoform-Dependent Cholesterol Regulation of HCN Channels
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Cholesterol is the major sterol component of all mammalian plasma membranes, and has been shown to regulate numerous ion channels. The hyperpolarization current, I_h, generated by the hyperpolarization-activated cyclic AMP-dependent (HCN) ion channels is a major contributor to the pacemaking in cardiac and neuronal tissue. However, regulation of each human HCN channel isoform (HCN1-4) by membrane lipids, including cholesterol, has not been systematically examined. Cholesterol depletion in CHO-K1 cells by MβCD or Mevastatin or enrichment using a cholesterol-MβCD complex expressing HCN1 or HCN2 channels leads to a reduction in current densities as well as altering the kinetics of activation and deactivation. Immunohistochemistry suggests that there is no change in the distribution of these channels from "lipid raft" domains to "non-raft" domains as a result of cholesterol modification. Specific isoform differences between HCN1 and HCN2 could be detected in non-equilibrium state known as "hysteresis", examined using ramp protocols. HCN1 demonstrates hysteresis when voltage ramps of 600mV/s are applied, whereas hysteresis is observed in HCN2 channels only at much slower ramp speeds (eg. 150mV/s). However, while the degree of hysteresis is unchanged in HCN1 channels when membrane cholesterol levels are modified, hysteresis in HCN2 channels begins to be apparent at faster ramps speeds (300 mV/s) upon cholesterol depletion. Such isoform dependent differences in cholesterol regulation of HCN channels may contribute to their differential distribution and function in cardiac and neuronal tissue.

3838-Pos Board B566
Ivabradine Reduces α-Smooth Muscle Actin Expression, Proliferation and Collagen Production in Human Cardiac Fibroblasts
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Fibrosis, a hallmark of cardiac remodelling is associated with disruptions of the myocardial architecture resulting from the accumulation and excessive deposition of extracellular matrix (ECM). Ivabradine (IVA), a selective inhibitor of the pacemaker current, has shown to have beneficial effects on pathological remodelling including a reversal in fibrosis providing a potential treatment in preserving cardiac structure. However, whether IVA directly alters cardiac fibroblasts, which are key regulators of the ECM, have yet to be investigated. The effects of 4 weeks treatment with IVA (30nM and 1μM) of left ventricular fibroblasts isolated from a patient with dilated cardiomyopathy on α-smooth muscle actin (α-SMA) expression, proliferation and collagen production, properties that are important in fibroblast-driven myocardial remodelling were assessed. α-SMA, a marker of activated fibroblasts was reduced by IVA in a dose-dependent manner (in arbitrary units, control: 535 ± 41; IVA30nM: 363 ± 20; IVA1μM: 181 ± 36; n=4; p<0.05). Using the MTS assay, proliferation was significantly attenuated by 1μM IVA (control: 0.09 ± 0.011nm; IVA30nM: 0.09 ± 0.007nm; IVA1μM: 0.03 ± 0.002; n=4; p<0.001). Collagen I production was also significantly reduced by 1μM IVA (control: 1.05 ± 0.026; IVA30nM: 0.96 ± 0.03; IVA1μM: 0.69 ± 0.07; n=4; p<0.001). The expression of hyperpolarisation cyclic nucleotide-gated channel isoforms 2 and 4 were identified in the fibroblasts. In addition effects of IVA on TGF-β1 expression was measured in a rat model of heart failure. HF was induced by permanent coronary artery ligation. After 12 weeks, HF animals were treated either with IVA (HF-IVA) or saline for a further 4 weeks. TGF-β1 expression (sham: 0.26 ± 0.03; HF: 0.49 ± 0.02; HF-IVA: 0.34 ± 0.01; n=4; p<0.05) was up-regulated during heart failure but IVA normalised this to sham levels. Our results suggest that IVA directly affect cardiac fibroblasts and this is a potential mechanism through which IVA could affect maladaptive remodelling of the ECM.

3839-Pos Board B567
Do Acidic Residues in the Tri-Asp Motif of the CNGA3 S2 Domain Form Required Pairings with Positive Residues of the S1-S4 Bundle? Evidence from Day-Blind Dogs and Insights from a Molecular Model of CNGA3 S1-S6 with Md Simulations
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Cone CNGA3 homo-tetramers were expressed in tSA201 cells. Missense mutations were generated in a highly conserved region of S2 containing three Asp residues denoted as the Tri-Asp motif. This motif is conserved in all CNG channel subunits. We substituted Asp 231 with Asn which resulted in complete loss of channel function. This conserved missense mutation is similar to Asp267Asn in canine CNGB3 which is associated with the loss of cone function resulting in day-blindness in the dogs. Further substitutions of all acidic residues in the Tri-Asp motif were generated with Ile, Asn, Cys, or Glu. Cyclic GMP-activated current data from excised patches of cells expressing CNGA3 channels showed that any substitution of an Asp residue resulted in the loss of channel function. Further, Asp substitutions resulted in mislocalization of a fluorescent tagged subunit suggestive of improper folding and ER retention. Our results highlight a crucial role for the Tri-Asp motif in channel biogenesis as well as function. A homology model of the S1-S6 domains of the cCNGA3 channel was created, using Kv1.2/2.1 as a template, and relaxed in a membrane with molecular dynamics simulations. The model shows that the Tri-Asp residues are involved in salt bridge pairings with positive residues of S2-S4. We hypothesize that the canine Asp/Asn mutation alters the electrostatic equilibrium in the S1-S4 bundle. In previous Shaker channel studies, conserved acidic residues in the Tri-Asp motif have been implicated in monomer folding. We, therefore, suggest that loss of the side chain cross bridges in the missense dog mutation destabilizes electrostatic interactions impairing the monomer folding state during biogenesis.

3840-Pos Board B568
Hyperpolarization-Activated and Cyclic Nucleotide-Gated Channels (HCN) are Modulated by Nitric Oxide in Magnocellular Neurons of the Supraoptic Nucleus of Rats
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Magnocellular neurons (MNCs) of the supraoptic nucleus are responsible for the synthesis of vasopressin and oxytocin. Several studies have shown that nitric oxide (NO) can play an important role in the control of the excitability of these neurons, by a direct action on ion channels. Considering that HCN channels are important for the control of the excitability of MNCs, we aimed to analyze the effects of NO on this type of current (I_h). For this purpose, hypothalamic slices were obtained from male Wistar rats and patch clamp technique was used to record I_h currents. Results show that 500 μM L-Arginine (substrate of NO) produced a significant reduction in the I_h current (−12.3 ± 3 pA) in relation to control (−24.8 ± 1.3 pA; at - 130 mV, n = 6; P < 0.05). In contrast, 100 μM L-NAME (inhibitor of NO synthase) significantly increased I_h (from −26.5 ± 9.4 pA in control to −69.7 ± 15.8 pA; n = 6; P < 0.05). Inactive isomers (D-Arginine and D-NAME) had no effects, confirming nitrergic specificity. The effects of NO seems to involve s-nitrosylation, since ODQ (specific blocker of guanylyl cyclase) did not change the baseline I_h. On the other hand, NEM (blocker of s-nitrosylation - 300 μM) induced an increase in the current (−33.7 ± 10.1 pA vs −81.2 ± 13.4 pA, at −130 mV, n= 10, P < 0.05). Also, in the presence of NEM, L-Arginine had no effects on I_h (−43.4 ± 9.4 vs −40.6 ± 6.8 pA at −130 mV, n=10, p>0.05). In conclusion,

our results show that HCN channels are involved in the nitrergic modulation observed in magnocellular neurons of the supraoptic nucleus acting through s-nitrosylation mechanism.

3841-Pos Board B569

Identification of a Pseudotetrameric K^+-Selective CNG Channel in *Amphioxus*

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A K^+-selective cyclic nucleotide-gated (CNGK) channel is key for chemotaxis in sea urchin sperm; it translates the second messenger signal after chemoattractant binding into an electrical response. We identified genes homologous to the sea urchin channel in the genome of different species - from invertebrates to vertebrates.

We cloned the CNGK channel from the testis of *Amphioxus*; *Amphioxus*, a chordateis considered as evolutionary link between invertebrates and vertebrates. We raised polyclonal antibodies to localize the channel in tissue and identified the channel in the flagellum of *Amphioxus* sperm. We analyzed the properties of the channel by functional expression and its physiological function by motility experiments of swimming sperm cells. Surprisingly, in contrast to the sea urchin CNGK channel, the *Amphioxus* CNGK channel seems to prefer cAMP over cGMP. In conclusion, signal transduction schemes in different sperm species display considerable diversity.

3842-Pos Board B570

A Family of HCN Channel Homologs in Bacteria

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HCN channels belong to the superfamily of six transmembrane domain voltage-gated ion channels. Functionally, these channels activate upon membrane hyperpolarization and carry an inward current that is weakly selective between potassium and sodium and can be modulated by cyclic nucleotides. At the structural level, insight into these channels is limited to a crystal structure of the intracellular C-linker and cyclic nucleotide binding domain, which assembles into a tetramer. To advance our structural understanding of HCN channels, we investigated bacterial homologs of HCN channels as candidates for large-scale expression and future structural studies. Using a BLAST search of the bacterial genome database we identified different homologs, which have a sequence identity of 24-32% with the human HCN2 channel. Using a C-terminal fusion with green fluorescent protein (GFP) we investigated whether the bacterial HCN homologs could be expressed in E. coli. We identified homologs that could be expressed at a whole-cell fluorescence level of ~40% compared to KcsA-GFP as a positive control. Next, we conducted detergent screening using fluorescence size exclusion chromatography (FSEC). We found that dodecylmaltoside or undecylmaltoside are suitable detergents to solubilize bacterial HCN channels in a tetrameric monodisperse state.

In parallel, we investigated the functional properties of the bacterial HCN channel homologs using expression of C-terminal GFP fusions in Xenopus oocytes. Using cell-attached patch recordings we observed in a minority of patches a current that may closely resemble the kinetics of the invertebrate spHCN channel as clear current inactivation is observed at most hyperpolarizing potentials. Confocal microscopy demonstrates marked fluorescence just below the oocyte membrane, indicating that trafficking to the oocyte cell membrane may be compromised and limits our success in obtaining patches for detailed functional characterization.

Together, our biochemical characterization paves the way for large-scale production and crystallization screening.

Intracellular Channels

3843-Pos Board B571

Functional Coupling of the Mitochondrial BKCa Channel to the Respiratory Chain

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Potassium channels as present in the plasma membrane of various cells have also been found in the inner mitochondrial membrane. Potassium channels have been proposed to regulate the mitochondrial membrane potential, respiration, matrix volume and Ca^{2+} ion homeostasis. Also, it has been suggested that mitochondrial potassium channels participate in ischemic preconditioning and neurodegenerative disorders.

In our study single channel activity of a large conductance Ca^{2+}-regulated potassium channel was measured by patch-clamp of mitoplasts isolated from an astrocytoma cell line. Mitoplast were prepared by addition to a hypotonic solution causing unfolding of the cristae of the inner membrane and consequently breaking of the outer membrane. Isotonicity was restored by adding a hypertonic solution. A potassium selective current was recorded with a mean conductance of 290 pS in symmetrical 150 mM KCl solution. The channel was activated by Ca^{2+} at micromolar concentrations and inhibited irreversibly by iberiotoxin, an selective inhibitor of the BKCa channel. Additionally, we showed that substrates of the respiratory chain like succinate decrease the activity of the channel. The effect was abolished by cyanide and antimycine, being inhibitors of respiratory chain.

Our findings indicate that mitochondrial large conductance Ca^{2+}-regulated potassium channels with properties similar to the surface membrane BKCa channel are present in human astrocytoma mitochondria and can be stimulated by redox status of the respiratory chain.

More details: Bednarczyk et al., (2013) PLoS ONE 8(6): e68125. doi:10.1371/journal.pone.0068125.

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3844-Pos Board B572

Electrophysiological Characterization of the Activity and Regulation of the Mitochondrial Calcium Uniporter

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Mitochondrial calcium uptake is present in all eukaryotic living organisms and represents a critical point because it is implicated in highly sophisticated processes that have multiple consequences for the cells' survival or death.

Ca^{2+} uptake by energized isolated mitochondria was directly measured for the first time half a century ago (Vasington and Murphy, 1962), but the molecular identity of the pore-forming subunit of the Ca^{2+} mitochondrial uptake protein complex MCU (mitochondrial calcium uniport), became available only in 2011, thank to the combination of bioinformatics, cell biology and the application of planar lipid bilayers experiments using recombinant MCU protein (De Stefani *et al*., 2011). Today, it is clear that the channel responsible for Ca^{2+} uptake is an inner mitochondrial membrane protein that works as a highly selective channel which can be inhibited by ruthenium red and gadolinium (Kirichok *et al*., 2004, De Stefani *et al*., 2011).

The application of electrophysiology in addition to cell biology techniques opens new perspectives to elucidate the physiological and pathological relevance of MCU and to directly study the role of numerous putative regulators/additional components of the MCU. For example, recent evidence shows that MCU is able to form hetero-oligomers with a protein, called MCUb, which acts as a dominant-negative pore forming subunit (Raffaello *et al*., 2013). Here we report further biophysical characterization of recombinant MCU studied in BLM experiments where possible regulatory factors are not present. Furthermore, we describe our studies using molecules, shown to affect calcium uptake in intact cells, like MICU1 (Perocchi *et al.,* 2013). Given that regulation of MCU activity in intact cells by MICU1 however is controversial (Mallilankaraman *et al*., 2012a/b; *Csordas et al., 2013*), our results provide an important, direct evidence in favor of direct regulation of channel activity.

3845-Pos Board B573

The Open State of Human VDAC Isoforms Compared through MD Simulations

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Voltage dependent anion channel (VDAC) is the pore-forming protein of outer mitochondrial membrane. In mammals three isoforms exist: VDAC1, VDAC2, VDAC3 (1). The VDAC1 is the most abundant (2) and studied isoform, the only one whose 3D structure was solved at high resolution. All-atom MD